Protein-Sized Bright Fluorogenic Nanoparticles Based on Cross-linked Calixarene Micelles with Cyanine Corona

Ievgen Shulov, Roman V. Rodik, Youri Arntz, Andreas Reisch, Vitaly I. Kalchenko, and Andrey S. Klymchenko*

Abstract: The key challenge in the field of fluorescent nanoparticles (NPs) for biological applications is to achieve superior brightness for sizes equivalent to single proteins (3-7 nm). We propose a concept of shell-cross-linked fluorescent micelles, where PEGylated cyanine 3 and 5 bis-azides form a covalently attached "corona" on micelles of amphiphilic calixarene bearing four alkyne groups. The obtained monodisperse NPs of 7 nm size increase their fluorescence quantum yield as a function of viscosity reaching 15% in glycerol. In the on-state they are ~2-fold brighter than quantum dots (QD-585), being the smallest PEGylated organic NPs of this high brightness. FRET between cyanine 3 and 5 cross-linkers at the surface of NPs suggests their integrity in physiological media, organic solvents and living cells, where they rapidly internalize showing excellent imaging contrast. Calixarene micelles with cyanine corona constitute a new platform for development of protein-sized ultrabright fluorescent NPs.

The research on fluorescent nanoparticles (NPs) is growing exponentially, because they are platforms for the fabrication of ultrabright multifunctional devices for imaging, diagnostics and therapy.[1] The most established examples of fluorescent NPs are quantum dots (QDs)[2] and dye-doped silica NPs,[3] which are of inorganic nature. Their typical hydrodynamic size is ~20 nm, although for cellular imaging with minimal perturbation of biomolecular processes, ideal NPs should be close to the size of proteins (3-7 nm). The solution is to assemble ultra-small organic fluorescent NPs from dyes. The large dye ensemble (~100 units) would ensure high fluorescence brightness comparable to QDs, while their organic content would make them biocompatible, non-toxic and eco-friendly. The area of organic NPs for bioimaging develops very rapidly. Here, one should mention conjugated polymer NPs,[4] dye-loaded polymer NPs,[5] DNA nanostructures,[6] and dye-based NPs,[7] including those exploiting aggregation induced emission.[5b, 8] However, obtaining bright and stable fluorescent NPs of 5-10 nm hydrodynamic diameter remains a challenge. Moreover, dyes tend to lose fluorescence due to aggregation-caused quenching (ACQ).[5c, 8-9] The key to both small size and minimized ACQ is the superior control of self-assembly of organic dyes with engineered inter-fluorophore distance and orientation. This could be achieved in micellar NPs, very small structures of 5-10 nm self-assembled from fluorescent amphiphiles. Though micellar assembly is very well-established,[10] only few examples in literature utilized this strategy to obtain fluorescent micellar NPs, notably using perylene diimides,[11] BODIPY[12] and aggregation induced emission[13] dyes. However, several key problems remain to be addressed so far in this field. First, aggregation into micelles usually produces broad emission with relatively low efficiency due to ACQ. Second, these micelles should be polymerized in order to prevent their disintegration and interaction with lipid structures of the cells.[12b, 14]

Presently, we propose a concept of protein-sized fluorescent NPs based on calixarene micelles that are shell-cross-linked by fluorescent bi-functional dyes *via* Cu-catalysed "click" chemistry. In one recent report, a surfactant bearing three acetylene groups was polymerized using non-fluorescent cross-linker, while the non-reacted alkynes were further modified with fluorescent mono-functional dyes.[15] In our strategy, we designed fluorescent cross-linkers to enable both micelle cross-linking and introduction of the fluorescent units. To this end, we selected cyanine 3 and 5 dyes, because of their excellent optical properties[16] and symmetric structure (Figure 1). They were modified with two azide groups for crosslinking of the micelle and two hydrophilic PEG(8) chains to create its biocompatible shell. We call this design "cyanine corona", similarly to well-known protein corona that modifies the surfaces of a variety of NPs.[17] As a reactive surfactant, we designed amphiphilic calixarene. Owing to the persistent conical shape, calixarenes are powerful building blocks for micelles,[18] nanoparticles, nano-cages, supramolecular polymers, etc.[19] They already found applications as antiviral, bactericidal and anticancer agents[20] as well as sensors[21] and vehicles for gene delivery.[22] Here, using a calixarene amphiphile bearing four alkyne groups, we prepared 7 nm micellar NPs shell-cross-linked with cyanine corona (Figure 1). They are the first protein-sized NPs combining fluorogenic response to viscosity with superior brightness compared to quantum dots (QD-585).

The calixarene amphiphile CX8TP at 150 μM concentration in aqueous solution (75 mM sodium sulfate) forms micelles of 5 nm according to dynamic light scattering (DLS) (Figure S1). These micelles were further reacted with 2 eq of the cross-linkers Cy3L or Cy5L in the presence of copper catalyst for 24h at 30 °C. Then these reaction mixtures were dialyzed and their pick absorbance was compared to that before the dialysis. We found that after the reaction only a fraction (≤50%) of the cross-linkers was removed, whereas the control samples without added Cu-catalyst lost nearly all cross-linkers after the dialysis (Figure 1A). These results provide an evidence for the successful cross-linking of micellar NPs with Cy3L and Cy5L. The absorption data suggested the cross-linking yields of 59 and 49% for Cy3L and Cy5L dyes, respectively. Moreover, the IR spectrum of the

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cross-linked micelles (CX/Cy3L) showed disappearance of the alkyne peak originally observed in CX8TP, which confirmed successful cross-linking (Figures S2). The absorption spectra of cross-linked micelles showed significant increase in the short-wavelength shoulder compared to free Cy3L and Cy5L dyes in water (Figure 2A). Appearance of this shoulder is an indication of the inter-fluorophore interaction, resulted from confinement of cyanine dyes grafted to calixarene micelles.

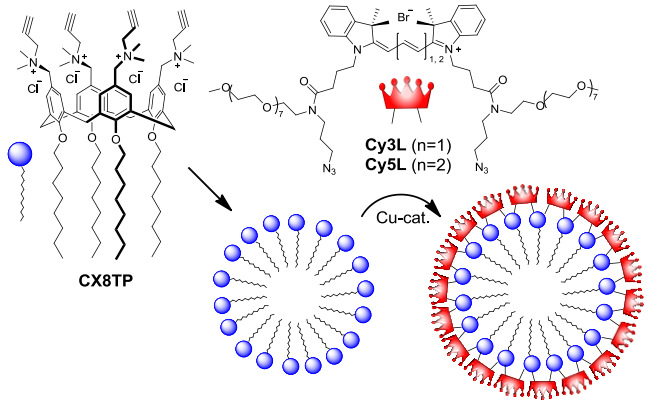

Figure 1. Concept of shell-crosslinking of calixarene micelles with cyanine corona.

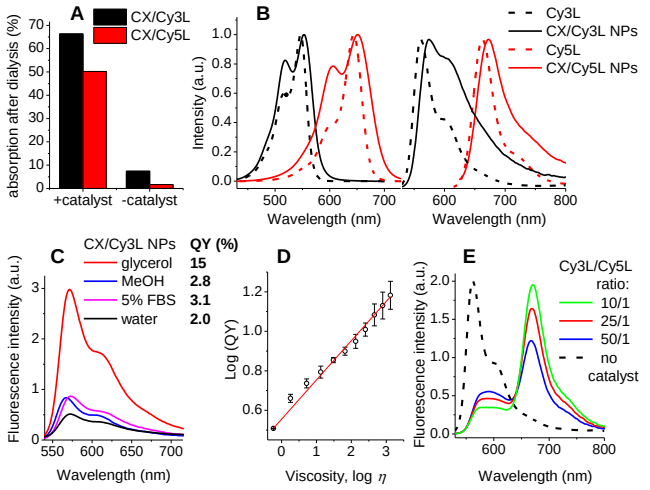

Figure 2. Absorption and fluorescence properties of cross-linked micelles. (A) Pick absorbance ratio after vs. before dialysis of dye cross-linkers and CX8TP (CX) micelles with and without Cu-catalyst. (B) Absorption and fluorescence spectra in water of free cross-linker dyes and the corresponding cross-linked micelles after dialysis. (C) Fluorescence spectra of Cy3L cross-linked micelles in different media. Excitation wavelengths for Cy3L and Cy5L were 520 and 605 nm, respectively. (D) Dependence of fluorescence quantum yield (QY) on viscosity (η). (E) Fluorescence spectra of FRET micelles prepared at different Cy3L (donor) to Cy5L (acceptor) ratio in water.

The dialyzed cross-linked CX/Cy3L micelles displayed a hydrodynamic diameter of 7 nm according to DLS (Figure S1) and zeta potential of 31±5 mV. Their transmission electron microscopy (TEM) showed remarkably homogeneous particles of 6.5±0.5 nm (Figures 3A and S3). Atomic force microscopy (AFM) in water confirmed a homogeneous population of NPs with the average height of 7±1 nm (Figures 3B and S5). Micelles based on Cy5L showed the same size according to AFM (Figures S4 and S5). The observed 2-nm increase in the NPs diameter after the cross-linking is clearly related to the additional corona-like cyanine shell of ~1-nm thickness (Figure 1).

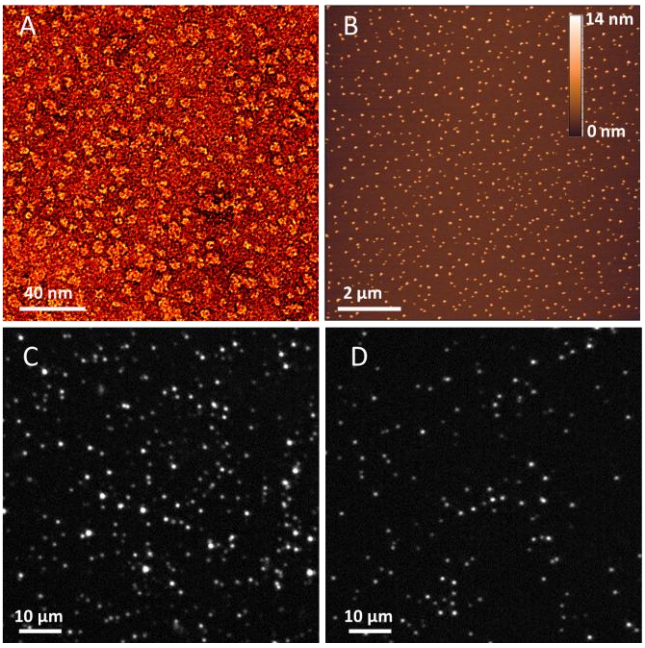

Figure 3. Single particle properties of fluorescent micelles. TEM (A) and AFM (B) images of shell-cross-linked CX/Cy3L micelles. (C-D) Wide-field fluorescence images of the same micelles deposited on glass in glycerol (C) and QD585 deposited on PEI/glass surface (D). The laser power density was 10 W cm^{-2} at 532 nm.

Importantly, the obtained micelles remained fluorescent, though their emission bands were red shifted and broadened compared to the free dyes Cy3L and Cy5L (Figure 2B). Moreover, the fluorescence quantum yield of the cross-linked micelles was lower compared to the free dye, namely 2 vs 13% for Cy3L and 0.1% vs 40% for Cy5L. This decrease is clearly linked to the ACQ, commonly observed for dye assemblies.[5c, 8, 23] In methanol, QY of CX/Cy3L micelles was also low compared to the free dye (Figure 2C), indicating that the micelles did not disintegrate. Strikingly, the fluorescence intensity of Cy3L micelles jumped many-fold in glycerol with respect to water with QY reaching 15% (Figure 2C). In glycerol-methanol mixtures, QY values depended on viscosity according to the Forster-Hoffman[24] equation (Figs. 2D and S6). The mean fluorescence lifetime also increased drastically from 0.15 ns in water and 0.16 ns in methanol to 1.27 ns in glycerol (Table S1). The absorption and excitation spectra in glycerol were similar to those in methanol showing a characteristic short-wavelength shoulder of emissive aggregates (Figure S7). The first reason for the fluorescence enhancement and the lifetime growth is that viscosity could decrease quenching by internal rotation in the grafted cyanine dyes.[25] Second, glycerol should decrease the mobility of the cyanines at the surface and thus decrease their collisional quenching. Interestingly, ~2-fold increase in the fluorescence intensity was also observed for CX/Cy3L micelles

in water with 5 vol% of serum (Figure 2C). The latter contains proteins and lipoproteins that could produce effects similar to those of glycerol, but at lower extent. To the best of our knowledge this is the first demonstration of fluorogenic nanoparticles that light up in viscous environment. The existing examples are limited to the turn-on response of aggregates produced by their disassembly.[12b, 14] Here, it is an intrinsic property of our micelles to sense the environment, which could be interesting for development of fluorogenic probes for bioimaging.[26]

To further evaluate single particle brightness, our NPs and quantum dots (QD-585) immobilized on the glass surface were compared by wide-field fluorescence microscopy. Strikingly, the brightness of our 7-nm NPs was ~2-fold higher than that of QD-585 (~22 nm diameter)[27] (Figures 3B,C and S8). The estimated brightness of these QDs when excited at 532 nm is ε × QY = 310 000 M^{-1} cm^{-1} × 0.67 = 2.1 × 10^5 M^{-1} cm^{-1},[27] which means that the brightness of CX/Cy3L NPs is ~4 × 10^5 M^{-1} cm^{-1}. Taking the aggregation number 40 for a parent calixarene amphiphile CX8,[18] and the cross-linking reaction yield of 59%, the expected number of Cy3L per micelle is 80 × 2 × 0.59 = 47. Then, the theoretical brightness of our NPs in glycerol at 532 nm excitation is 47 × 100 000 M^{-1} cm^{-1} × 0.15 = 7.1×10^5 M^{-1} cm^{-1}. This value is close to the experimental value obtained by microscopy. In water, micelles deposited on glass showed somewhat lower brightness than in glycerol, though many of them were as bright as QDs (Figure S9), possibly due to the contact with the rigid glass surface. Finally, fluorescence correlation spectroscopy (FCS) suggested the presence of 5.9 nm NPs in water with brightness equivalent of 52 Cy3L dyes (Table S2), matching well the theoretical number (47) of dyes per micelle.

We also explored a possibility to prepare multi-component shell-cross-linked micelles using both Cy3L and Cy5L cross-linkers, which would serve as FRET donor and acceptor, respectively. The relative intensities of the absorption peaks of Cy3L and Cy5L (Figure S10) suggested that for formulations with the donor/acceptor ratios 10, 25 and 50, the actual ratio after cross-linking was relatively close, 18, 48 and 79, respectively. The strong FRET signal was observed already at donor/acceptor ratios of 50, and further increased for 25 and 10 ratio, which can be seen from the gradual increase in the relative intensity of the acceptor (Figure 2E). It should be noted that with decrease in the donor/acceptor ratio, the growth of the acceptor intensity was somewhat stronger than the loss of the donor intensity. We expect that in this case, FRET could compete with self-quenching phenomena within the donor dyes, as it was shown for dye-doped silica NPs.[28] By contrast, no FRET signal was observed without Cu-catalyst. These results provide evidence for successful grafting of both cross-linkers within the same micelle and the possibility to control the grafting ratio. The high FRET efficiency at 50/1 ratio (69%) shows that these NPs appear as efficient light-harvesting system,[29] where a single acceptor collects energy from many donors within a 7-nm space of the micelle. As FRET is sensitive to the donor-acceptor distance, it can be used to probe integrity of NPs.[30] Remarkably, in methanol or in water with 5% serum the acceptor fluorescence intensity remained higher than that of the donor (Figure S11), indicating that the FRET was preserved. The latter indicates that shell-cross-linking of calixarene micelles ensured their stability in organic solvent and biological medium.

Finally, to check their applicability to bioimaging, CX/Cy3L NPs were incubated with HeLa cells. NPs appeared inside the cells as bright dots, showing a distribution typical for endosomes and lysosomes (Figure 4C). By contrast, without NPs no fluorescence was detected, while addition of non-cross-linked micelles (Cy3L with CX8TP without catalyst) gave diffuse intracellular fluorescence (Figure 4A,B). Thus, without cross-linking, free Cy3L dye internalized and distributed over the cytoplasm, whereas cross-linked micelles probably remained intact inside the cells. Remarkably, our FRET NPs excited at 561 nm showed intracellular dotted emission co-localized in the donor (Cy3) and FRET acceptor (Cy5) channels (Figure 4D-F). By contrast, very poor emission in the Cy5 channel was observed for non-cross-linked micelles, where Cy3L and Cy5L cannot undergo FRET (Figure S12). Thus, our FRET experiments provided direct proof of the integrity of the cross-linked NPs inside the cells.

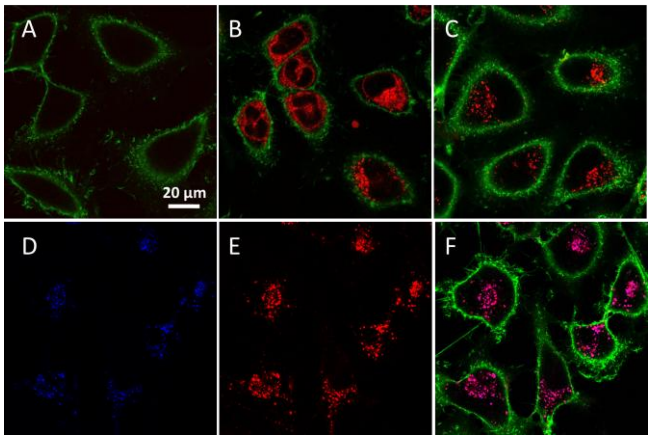

Figure 4. Fluorescence confocal imaging of HeLa cells incubated with fluorescent micelles for 3h at 37°C. Control cells without micelles (A); cells incubated with non-cross-linked (B) or cross-linked (C) CX/Cy3L micelles. Green corresponds to plasma membrane staining with WGA-Alexa Fluor488 (50nM), while red corresponds the micelles. Concentration of Cy3L in free form and in cross-linked micelles was 0.5 µM. (D-F) Images of HeLa cells incubated with FRET micelles (Cy3L/Cy5L ratio 10/1). (D) Signal from the FRET donor recorded at 576-640 nm (Cy3 channel). (E) Signal from the FRET acceptor recorded at 650-750 nm (Cy5 channel). (F) Merged images of donor, FRET and membrane marker channels. Excitation wavelength for the FRET micelles and WGA-Alexa Fluor488 was 561 and 488 nm, respectively.

In summary, we propose a concept of fluorescent shell-cross-linked micelles presenting very small size, fluorogenic behaviour in viscous media and high brightness. It is based on PEGylated cyanine bis-azides, which form covalently attached "corona" on micelles of calixarene amphiphiles bearing four alkyne groups at the polar heads. We obtained 7-nm NPs that increase their fluorescence efficiency from 2% in water up to 15% in glycerol. Microscopy shows that they are ~2-fold brighter than quantum dots (QD-585 at 532 nm excitation). Remarkably, superior brightness vs QDs is achieved for >3-fold smaller hydrodynamic diameter, which is a crucial advantage. Although theoretical and experimental brightness of our NPs (7.1 × 10^5 and ~4 × 10^5 M^{-1} cm^{-1}) is lower than that of conjugated polymer

NPs (P-Dots),[4] their size including PEG shell is significantly smaller (7 vs 10-20 nm). They present excellent stability in aqueous and organic media, and enter readily the cells showing high signal to noise ratio without dye leakage. Thus, we propose a new platform for developing bright protein-sized responsive nanoparticles for bioimaging.

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Keywords: fluorescence • cyanines • calixarenes • click chemistry • nanoparticles

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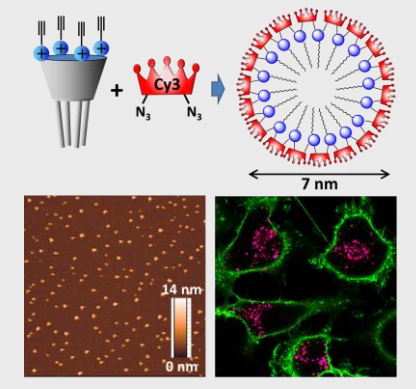

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